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TITLE: Randomized Trial of Neuroprotective Effects of Erythropoietin in Patients Receiving Adjuvant Chemotherapy for Breast Cancer: Positron Emission Tomography and Neuropsychological Study

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14. ABSTRACT

Animal Research Study Amendment An amendment to the study was initiated in April 2005 to include animal experiments. As per published literature, proinflammatory cytokines play a role in the pathogenesis of cognitive dysfunction. The experiments were designed to assess the cytokines before and after chemotherapy in a rat model. We have established an experimental animal model to study chemotherapy-induced cognitive dysfunction observed in the clinical setting. In this model administration of four weekly doses of clinical chemotherapeutics, i.e., the combination of adriamycin and cytoxan, results in impaired memory function in rats. We propose to further characterize the mechanisms of this cognitive dysfunction by molecular genomics approach. We will analyze global gene expression in the hippocampi from treated vs. control rats using the microarray methodology. This analysis will allow us to identify genes whose expression is altered by the chemotherapeutic treatment. Subsequently, expression of these genes will be analyzed by real time RT-PCR to confirm the microarray results, to precisely quantify the differential expression of these genes, and to further streamline the selection of putative genes that underscore the memory impairment. This study will provide important information for future, more clinically oriented endeavors to determine the correlation between the polymorphism of these genes and the susceptibility of patients to chemotherapy-induced cognitive dysfunction.

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Introduction

In the United States approximately 60-80% of patients diagnosed with breast cancer will receive adjuvant chemotherapy. Of these patients more than 30% will experience short-term and long-term cognitive impairment (e.g., problems with memory and concentration) for at least 1-2 years after completion of chemotherapy. Despite the effects cognitive impairment can have on a patient's quality of life very few studies have been conducted to learn more about this side effect.

This study aimed to evaluate the pathophysiology of cognitive dysfunction in patients receiving adjuvant chemotherapy with Adriamycin and cyclophosphamide for breast cancer using [^{15}O] water PET scans and neuropsychological tests.

The intent of the study was to enroll a total of 24 eligible patients with early stage breast cancer who were candidates for adjuvant chemotherapy. It was planned for each patient to undergo [^{15}O] water PET scans at baseline and after completing 4 cycles of adjuvant chemotherapy to measure the differences in regional blood flow of the brain during working memory. Neuropsychological tests were to be done to determine attention, speeded processing, memory, and executive functions outside of the [^{15}O] water PET scans.

In April 2004 this study was amended due to published data regarding the safety of Epoetin alfa (EPO). This data showed that patients with normal hemoglobin levels who receive treatment with EPO have increased morbidity and mortality. Due to this information the Investigators were prompted to change the protocol to look at pre-post chemotherapy changes in the brain with PET scan, but without treating patients with EPO. Therefore the protocol aims to understand the pathophysiology of cognitive dysfunction without studying the role of EPO. The final report for this study was submitted in September 2006.

An amendment to the study was initiated in April 2005 to include animal experiments. As per published literature, proinflammatory cytokines play a role in the pathogenesis of cognitive dysfunction. The experiments are designed to assess the cytokines before and after chemotherapy in a rat model, as explained below. We have established an experimental animal model to study chemotherapy-induced cognitive dysfunction observed in the clinical setting. In this model administration of four weekly doses of clinical chemotherapeutics, i.e., the combination of adriamycin and cytoxan, results in impaired memory function in rats. We propose to further characterize the mechanisms of this cognitive dysfunction by molecular genomics approach. We will analyze global gene expression in the hippocampi from treated vs. control rats using the microarray methodology. This analysis will allow us to identify genes whose expression is altered by the chemotherapeutic treatment. Subsequently, expression of these genes will be analyzed by real time RT-PCR to confirm the microarray results, to precisely quantify the differential expression of these genes, and to further streamline the selection of putative genes that underscore the memory impairment. This study will provide important information for future, more clinically oriented endeavors to determine the correlation

between the polymorphism of these genes and the susceptibility of patients to chemotherapy-induced cognitive dysfunction.

Body

Task 1. Study the baseline cognitive function **Completed**

- Five patients with early stage breast cancer receiving adjuvant chemotherapy have been enrolled in this study. One patient was withdrawn due to the inability to complete the baseline PET scan. The patient became claustrophobic during the PET scan, therefore, the scan was not completed.
- Baseline cognitive function assessments with neuropsychological measures have been completed by each patient.
- The baseline study of regional blood flow of the brain using [^{15}O] water PET scans during working memory has been completed by each patient.

Task 2. To study the cognitive function after 4 cycles of chemotherapy with [^{15}O] *water Positron Emission Tomography (6-12 months)* **Completed**

- Each patient completed PET scans and neuropsychological measures 2-4 weeks after the completion of 4 cycles of AC.

Task 3. Analysis of the data and writing of the final report (12-18 months) **Completed**

- All data is maintained in a secure database. The human study data will not be analyzed due to the small sample size.

Task 4. Animal Study **Completed Prior to Amendment (02/2007)**

- Baseline cytokine assay in the peripheral blood has been completed.
- Animals were treated with Adriamycin and cytoxan every two weeks for 4 cycles.
- Cytokines in the blood were measured weekly while the animals were being treated with chemotherapy.
- Analyzed the brains of chemo-treated rats by histology.

Task 5. Animal Study - Identifying target genes involved in chemotherapy-induced cognitive dysfunction

- Treat rats with weekly intraperitoneal injections of adriamycin/cytoxan for four weeks, and evaluate memory impairment by behavioral testing.
- Collect brain tissue, extract and purify hippocampal mRNA.
- Perform gene expression profiling using microarrays to identify genes that are differentially expressed in control vs. treated hippocampi.
- Confirm differential expression of the identified genes by quantitative RT-PCR.

Key Research Accomplishments

Proinflammatory cytokine expression as a possible mechanism of chemotherapy induced cognitive dysfunction

In this preliminary study we tested the effect of commonly used chemotherapeutics on rats to elucidate the mechanisms of chemotherapy-induced brain damage leading to

cognitive dysfunction, and ultimately, to understand how these mechanisms can be controlled for therapeutic purposes. We used ten month old Sprague-Dawley female rats (retired breeders) injected intraperitoneally with a combination of adriamycin and cytoxan (1:10 w/w) as an experimental system. The initial experiments were carried out to establish non-lethal and non-morbid dosage of the chemotherapeutics. Three groups of rats were treated weekly with the following doses of adriamycin/cytoxan (mg/kg): (A) 2.5/25, (B) 5/50 and (C) 10/100. Control group was injected with saline only. All rats (n=4) in group B and C died after three and two injections, respectively, but no mortality was observed in group A. Over four weeks of the experiment the body weight increased by approximately 2 and 4% in the A vs. control group, respectively indicating no significant morbidity. Therefore, the dosage of 2.5 mg/kg of adriamycin and 25mg/kg of cytoxan was used in the subsequent experiments. This treatment is further referred to as "chemo-treatment".

To address Specific Aim 1 we measured the level of proinflammatory cytokines in the peripheral blood of the treated vs. control animals. Blood samples were collected 1, 2 and 7 days after each injection, and the level of TNFα was determined by ELISA. The baseline level of the cytokine was 6.8±1.8 pg/ml as measured in saline injected rats (control group), and did not change in the course of the experiment. Also, chemo-treatment did not significantly affect TNFα in the blood at any time point. These negative results indicate that the mechanisms of the drug action may not be mediated by peripheral cytokines. Therefore, before proceeding to Specific Aim 2 to assess changes in brain cytokine expression we decided to determine whether our chemo-treatment paradigm affects brain function.

We performed several behavioral tests to assess the effects of chemo-treatment on cognitive function of the rats. We tested the animals for locomotive activity using the open field test. No differences between control and treated rats at the beginning or at the end of the experiment were detected. Also, chemo-treatment did not change the exploratory activity of the rats as detected by the novelty test. After establishing this we assessed the memory function of the animals using the passive avoidance test. As shown in Fig. 1 this test revealed a significant impairment of cognitive function.

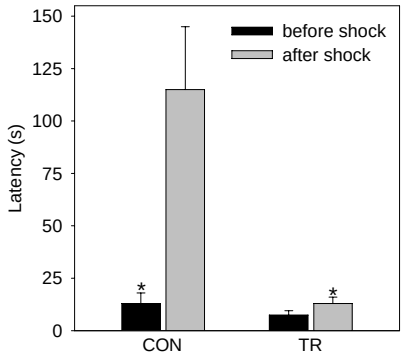

Fig. 1. Performance of chemo-treated (TR) and control (CON) rats in passive avoidance test. The animals were tested one week after the last dosing. Briefly, a rat was placed in illuminated chamber, and the latency of entering into dark chamber was recorded. Upon entering dark chamber electric shock was administered. Twenty four hours later, the rat was placed again in illuminated chamber, and the latency of entering the dark chamber was recorded for up to 180 s. Results are means ± SEs.
** values significantly different from electrically shocked control rats ($p \leq 0.05$)*

No significant difference in the latency of entering in the dark chamber between control and treated groups was evident before the electric shock was applied. However,

the latency after the electric shock increased profoundly in the control group, but not in the chemo-treated group. Because there were no locomotor and no exploratory deficiencies in the treated animals, this lack of increased latency demonstrates that chemotherapy impaired memory function as the animals did not remember the electric shock experience and did not avoid entering the dark chamber. Therefore, our chemo-treatment paradigm models cognitive dysfunction in patients undergoing chemotherapy. This experimental model provides a convenient system for further studies of the mechanisms of brain damage elicited by chemotherapy.

We also found no abnormalities in the blood brain barrier of chemo-treated animals using the trypan blue exclusion assay. We are currently in the process of analyzing the brains of chemo-treated rats by histology. Because the treatment impairs memory function of the animals we focus primarily on identifying cellular changes in the hippocampus.

In summary, we have demonstrated that chronic administration of clinical chemotherapeutics, i.e., the combination of adriamycin and cytoxan, severely impairs memory function in rats. This study provides an experimental animal model to study chemotherapy-induced cognitive dysfunction observed in the clinical setting.

Reportable Outcomes

Presentation

Invited Speaker, "Use of PET Scanning in Assessing the Pathophysiology of Cognitive Dysfunction: The Future of Supportive Therapy in Oncology an International Congress," Hamilton, Bermuda, March 13, 2003.

Presentation

"Cognitive Dysfunction in Adjuvant Breast Cancer Treatment," Mary Babb Randolph Cancer Center Research Retreat, Stonewall Jackson Resort, July 13-15 2005.

Poster Presentation

"The Effects of Adjuvant Chemotherapy for Breast Cancer on Cerebral White Matter and Cognitive Function: A Diffusion Tensor Imaging Pilot Study," American Society of Clinical Oncology Annual Meeting, May 15-18, 2005.

Presentation

"Chemotherapy-induced cognitive dysfunction in rats" by Kraszpulski M, James I, Zhang H-T, Krasowska A, Abraham J and Konat G., Nemacolin Woodlands Resort, September 25-26, 2006.

Presentation

"Experimental model of chemotherapy induced cognitive dysfunction" by Konat G.W., Kraszpulski M, James I, Zhang H-T, and Abraham J, 21st Biennial International Society for Neurochemistry Meeting, August 19-24, 2007.

The following individuals received grant funding for their work on this project:

- Jame Abraham
- Marc Haut

- Gregory Konat
- Alicia Krasowska

Conclusions

Accrual for this study began in April 2003 and five patients have been enrolled; with four patients being evaluable. We amended the original protocol to drop EPO and the study was temporarily stopped for accrual in April 2004. The site received permission from the DOD to resume enrollment in April 2005 after the amendment.

In the last three years, there has been a change to commonly used chemotherapeutic regimens in the adjuvant breast cancer setting. Many newer regimens contain a taxane, instead of an anthracycline or taxanes and anthracyclines are used together. The study was not originally designed to include patients receiving a taxane, therefore, enrollment to the study has been difficult. Unfortunately we were unable to meet the accrual goal of 24 patients.

However, the animal research has some exciting findings, as explained above. We have a very successful animal model for cognitive dysfunction now. We are really interested in pursuing the study of pathophysiology of cognitive dysfunction by identifying the candidate genes in animals which caused memory problems. Once we identify the candidate genes, then we can translate that information into humans to look for polymorphism in those genes in humans leading to cognitive dysfunction.